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A technological advance comparing epithelial lining fluid from different regions of the lung in smokers

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Small airways;
Smoking

Summary
Cigarette smoking causes inflammatory responses in the airways. However, not all smokers exhibit the development of airflow limitation. This study was designed to determine the implications of small airways inflammation in the development of airflow limitation in smokers by our newly explored method. Twenty-eight smokers (15 smokers without airflow limitation and 13 with airflow limitation) were included in this study. Levels of interleukin-8 (IL-8) and 8-isoprostane were measured in epithelial lining fluid (ELF) from central and peripheral airways separately collected using a bronchoscopic microsampling technique. 8-isoprostane levels in ELF from central or peripheral airways did not significantly differ between the two groups. However, these levels were markedly higher in peripheral than in central airways. Similarly, IL-8 levels in ELF from central airways did not significantly differ between the two groups. In smokers without airflow limitation, IL-8 levels were not higher in peripheral than in central airways. In contrast, in smokers with airflow limitation, IL-8 levels were significantly higher in peripheral airways. Moreover, in smokers with airflow limitation, 8-isoprostane levels in central or peripheral airways were not significantly correlated with FEV_1. However, IL-8 levels in peripheral airways were inversely correlated with FEV_1, though those levels in central airways were not. Thus our technique provides a novel method for ELF sampling from central or peripheral airways separately, and the preliminary evidence that support differences in oxidative stress and neutrophil chemotactic stimulus in these two locations.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major worldwide health problem that has an increasing prevalence and mortality.[1] The prevalence, morbidity, and mortality of COPD vary appreciably across countries, but in

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general are directly related to the prevalence of cigarette smoking. COPD is a disease state characterized by airflow limitation that is not fully reversible, usually progressive, and associated with an abnormal inflammatory response of the lungs. Indeed, one important pathological feature of COPD is chronic airway inflammation characterized by an influx of inflammatory cells — predominantly neutrophils, macrophages and CD8 + T lymphocytes — in the lumen and wall of the bronchial and bronchiolar airways and parenchyma.[2] One prevalent theory concerning the pathogenesis of COPD is of an abnormal balance between proteases and anti-proteases in the lung.[3] This theory proposes that increased numbers of neutrophils, activated by cigarette smoke, produce large amounts of proteases and oxidants responsible for lung destruction. Thus, increased oxidative stress, which can be defined as an increased exposure to oxidants and decreased antioxidant capacities, is widely recognized as a central feature of COPD.[4] Since increased oxidative stress leads to extensive cellular injury and damage, oxidants produced by pulmonary inflammatory cells play an important role in the pathophysiology of COPD. However, the initial inflammatory properties of this disease have not yet been fully elucidated.

Cigarette smoking causes inflammatory responses in the airways.[5] These changes include infiltration of inflammatory cells such as neutrophils and macrophages, and thickening of the airway walls with increased collagen deposition.[6] Such inflammatory and remodeling processes are believed to be related to the resultant obstructive changes in the airways. These smoking-related changes in the airways impose a major risk for the development of COPD. Stefano and coworkers have already demonstrated that airflow limitation is associated with severity of airway inflammation in smokers.[7] Moreover, it has been shown that smoking can progress airflow limitation, as evidenced by an increase in the rate of decline in forced expiratory volume in 1 s (FEV1).[8] In particular, it is well known that smoking-induced structural changes mainly originate in the peripheral airways.[9] However, not all smokers exhibit the development of airflow limitation, which suggest that various factors in each individual susceptible to cigarette smoking may modify lung function abnormalities.[10] On the basis of these findings, we hypothesized that differences in inflammatory properties of small airways may exist in smokers with and without airflow limitation. To test our hypothesis, a sensitive method to investigate characteristics of small airways will be required. In this study, to evaluate the properties of small airways inflammation in the development of airflow limitation, levels of interleukin-8 (IL-8) and 8-isoprostane were measured in epithelial lining fluid (ELF) from central and peripheral airways separately collected from smokers with and without airflow limitation using a bronchoscopic microsampling technique.

Methods

Subjects

This study prospectively enrolled 28 current smokers from the outpatient clinic of our institution, who underwent bronchoscopy to identify the cause of persistent cough or small peripheral nodules. All smoking subjects who agreed to address ELF samplings were randomly selected. Chest computed tomographic scans showed no abnormal diffuse interstitial infiltrates, and results of arterial blood gas analyses were normal. We divided these subjects into two groups based on a pulmonary functional viewpoint (15 smokers without airflow limitation ($FEV_1/FVC > 70\%$) and 13 smokers with airflow limitation ($FEV_1/FVC < 70\%$)). All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Bronchoscopic microsampling technique

The interval time from the last cigarette smoking in each subject was at least more than 24 h. ELF was obtained using a previously described bronchoscopic microsampling technique.[11,12] In brief, after premedication of the subject with atropine and pentazocine, and administering local anesthesia with aerosolized lidocaine hydrochloride, a flexible fiberoptic bronchoscope (BF-240, Olympus, Tokyo, Japan) was inserted in the trachea and, after flushing with air to minimize contamination of the samples, advanced into the target bronchus. Subsequently, the microsampling probe (BC-402C, Olympus, Tokyo, Japan) was then inserted through the channel of the bronchoscope. The probe consists of a 2.5-mm outer diameter polyethylene sheath and a 1.9-mm inner polyester fiber rod probe attached to a stainless steel guide wire. Bronchial microsampling techniques were performed in all subjects from the right bronchus intermedius (central airways). Next, a thin flexible fiberoptic bronchoscope (BF-P260F, Olympus, Tokyo, Japan) was inserted into the lung, and the microsampling probe (BC-401C, Olympus, Tokyo, Japan) was then inserted through the channel of the bronchoscope. This probe consists of a 1.8-mm outer diameter polyethylene sheath and a 1.1-mm inner polyester fiber rod. We obtained ELF from seventh or eighth lower lobe bronchioles under direct vision using this thin bronchofiber scope in the same subjects (peripheral airways). ELF was collected in each subject from the contralateral lung field of small peripheral nodules. The inner probe was advanced into the bronchial lumen slowly for 15 s to avoid injuring the bronchial wall. The inner probe was then withdrawn into the outer tube, and both were withdrawn together to avoid contamination.

Table 1 Clinical characteristics of study subjects

	Current smokers	
	Without airflow limitation	With airflow limitation
Subject no. (male/female)	15 (12/3)	13 (12/1)
Age (yr)	69 (9)	64 (14)
Smoking index (pack-yr)	48 (25)	49 (43)
FEV_1 (% predicted)	126 (21)	67 (18)[a]
Dlco (%)	93 (22)	85 (25)[a]

All values are presented as mean (SD).
Definition of abbreviations: FEV_1 = forced expiratory volume in 1 s, Dlco = diffusing capacity of carbon monoxide.
[a] $p < 0.01$ Compared with smokers without airflow limitation.

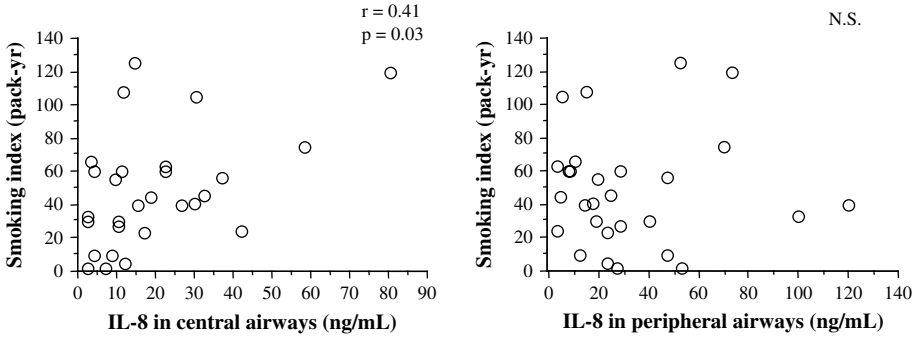

Figure 1 Correlations between smoking index and IL-8 levels in ELF from central or peripheral airways for all study subjects.

Thus, the same procedure was repeated three times. And then the inner probe was cut at 3 cm distal from its tip, and the wet probe was frozen at a −80 °C freezer until use. The diluted solution for measurements of biochemical constituents was prepared by adding 1 ml of saline to the tube containing the frozen probe and vortexed for 1 min. The probe was dried and weighed to measure the ELF volume recovered, and the dilution factor was calculated.

Measurement of biochemical constituents in ELF

Using the ELF sample processed as above mentioned, the concentration of 8-iso-prostaglandin F2α (8-isoprostane),[13] one of the major biomarkers of oxidative stress, was detected by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI). The assay for 8-isoprostane has 4 pg/mL detection limit, using antiserum that has 100% reactivity with 8-isoprostane. The concentrations of IL-8 were also measured using commercial ELISA kits (BioSource, Belgium). The detection limit for IL-8 was 8 pg/mL.

Statistical analysis

All values are presented as mean (SD) or median [range] depending on whether they were normally distributed. The unpaired *t*-test was used for comparisons of parametric data. When multiple comparisons of nonparametric data were made between groups, significant intergroup variability was first established by using the Kruskal—Wallis test. The Mann—Whitney *U*-test was then used for intergroup comparisons. The Wilcoxon signed-rank test was also used for comparisons of variables between central and peripheral airways. The significance of correlations was evaluated by determining Spearman's rank correlation coefficients. A *p*-value of less than 0.05 was considered significant.

Results

The clinical characteristics of 15 smokers without airflow limitation ($FEV_1/FVC > 70\%$) and 13 smokers with airflow limitation ($FEV_1/FVC < 70\%$) are shown in Table 1. The two groups were well matched for age and smoking index, though FEV_1 and Dlco were significantly lower in smokers with airflow limitation. We could safely accomplish bronchoscopic microsampling techniques in all study subjects. For all study subjects, smoking index was significantly correlated with IL-8 levels in ELF from central airways ($r = 0.41$, $p = 0.03$), but not with those levels from peripheral airways (Fig. 1).

8-isoprostane levels in ELF from central or peripheral airways did not significantly differ between the two groups (without airflow limitation: central 0.75 [0—4.4] ng/ml, peripheral 6.6 [0—19.6] ng/ml; with airflow limitation: central 0.43 [0—5.7] ng/ml, peripheral 3.7 [0—18.4] ng/ml) (Fig. 2). However, these levels were markedly higher in

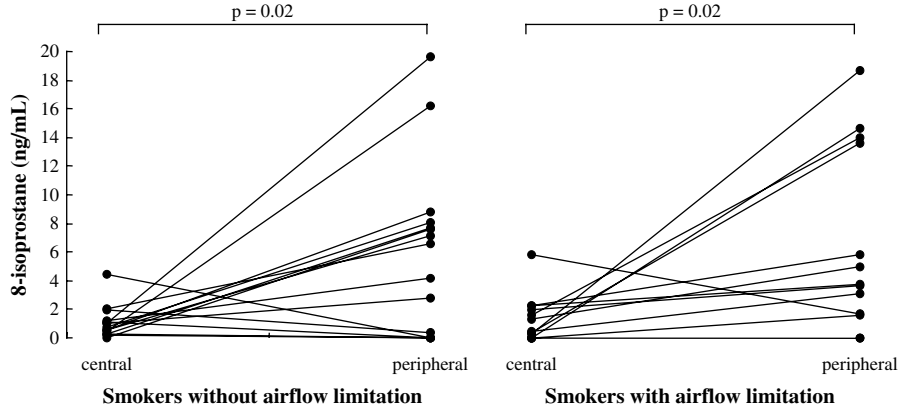

Figure 2 Comparisons of 8-isoprostane levels in ELF from central and peripheral airways in smokers with or without airflow limitation.

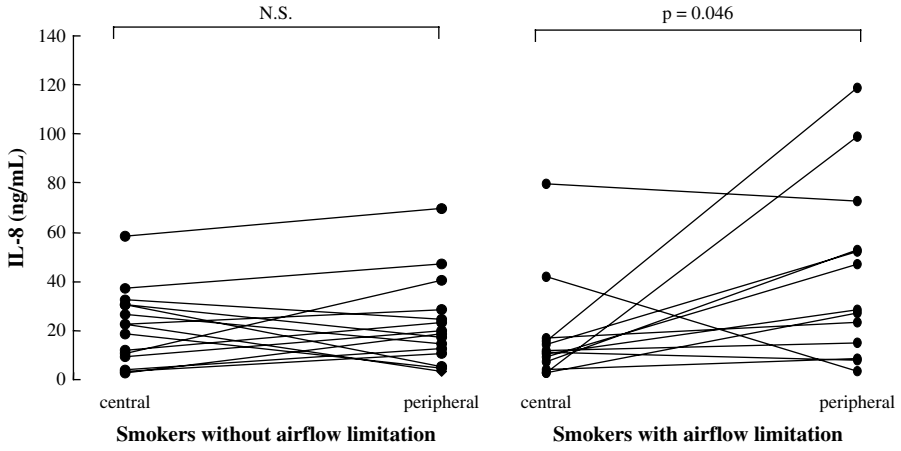

Figure 3 Comparisons of IL-8 levels in ELF from central and peripheral airways in smokers with or without airflow limitation.

peripheral than in central airways (without airflow limitation: $p = 0.02$; with airflow limitation: $p = 0.02$). Similarly, IL-8 levels in ELF from central airways did not significantly differ between the two groups (without airflow limitation: 22.3 [2.5—58.5] ng/ml; with airflow limitation: 11.2 [2.3—80.5] ng/ml) (Fig. 3). These levels in smokers without airflow limitation were not significantly higher in peripheral than in central airways. In contrast, these levels in smokers with airflow limitation were significantly higher in peripheral than in central airways ($p = 0.046$).

In smokers with airflow limitation, we evaluated the correlations between degree of airflow limitation and levels of IL-8 or oxidative stress. 8-isoprostane levels in central or peripheral airways were not significantly correlated with FEV_1 (Fig. 4). Similarly, IL-8 levels in central airways were not also correlated with FEV_1 (Fig. 5). However, these levels in peripheral airways were inversely correlated with FEV_1 ($r = -0.64$, $p = 0.026$).

Discussion

The bronchoscopic microsampling techniques have methodological advantages compared with sputum or bronchoalveolar lavage fluid sampling techniques by enabling quantitative analysis. These advantages encouraged us to utilize this technique to obtain ELF samples in human subjects and to assess the inflammatory markers quantitatively. Moreover, a recent clinical interest has been devoted to a sensitive method explored for early detection of small airways inflammation in smokers. From these viewpoints, we developed a new method to obtain ELF samples separately from central and peripheral airways using a thin fiberoptic bronchoscope. Using this method, we found that 8-isoprostane levels in ELF from central or peripheral airways did not significantly differ between smokers with and without airflow limitation, but that these levels were markedly higher in peripheral than in central airways in the two groups. 8-isoprostane is considered to be a reliable index of *in vivo* oxidative stress because they are structurally stable and are produced *in vivo*.[14] Many previous studies have demonstrated that increased concentrations of 8-isoprostane are a common feature of chronic inflammatory diseases.[15] In fact, elevated 8-isoprostane concentrations in induced sputum and exhaled breath condensate have been reported in patients with asthma, COPD, and interstitial lung disease.[16] These reports highlight the involvement of oxidative stress in various inflammatory lung diseases. However, in this study we found that elevated levels of oxidative stress may not be the sole mechanism responsible for the manifestation of airflow limitation in smokers.

The second novel aspect of this investigation is the finding that (i) IL-8 levels in ELF from central airways did not significantly differ between smokers with and without

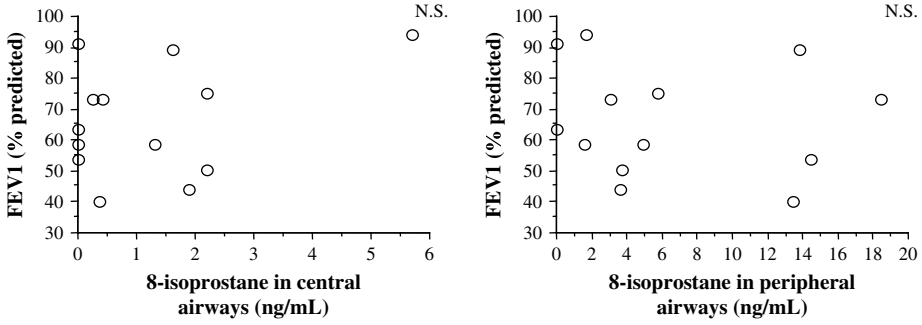

Figure 4 Correlations between 8-isoprostane levels in ELF from central or peripheral airways and FEV_1 in smokers with airflow limitation.

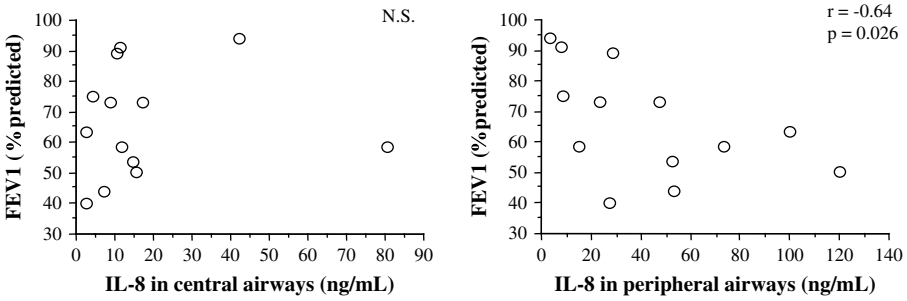

Figure 5 Correlations between IL-8 levels in ELF from central or peripheral airways and FEV_1 in smokers with airflow limitation.

airflow limitation, (ii) IL-8 levels in ELF from peripheral airways in smokers with airflow limitation, but not in smokers without airflow limitation, were significantly higher than those from central airways, and (iii) in smokers with airflow limitation, IL-8 levels in ELF from peripheral airways were associated with severity of airflow limitation. IL-8 is a cytokine synthesized by a variety of inflammatory cells in the lung, and a potent activator of neutrophils.[17] IL-8 induces superoxide anion release from neutrophils *in vitro*, and intravenous administration of IL-8 *in vivo* has been reported to induce accumulation of neutrophils in the lung. Therefore, expression of IL-8 may be critically important in the pathogenesis of smoking-induced lung diseases. In this study, we found a significant correlation between smoking index and IL-8 levels in ELF from central airways. However, we could not observe a significant correlation between smoking index and those levels from peripheral airways. These findings suggest that total amounts of cigarette smoking are associated with magnitude of neutrophilic inflammation in central airways, and that not all smokers demonstrated neutrophil chemotaxis into peripheral airways. Thus, neutrophil chemotaxis into peripheral airways may be, at least in part, responsible for the manifestation of airflow limitation in smokers. In the present study, we found that concentration of IL-8 in peripheral airways of smokers with airflow limitation was higher than those in central airways, and that peripheral IL-8 levels were associated with airflow limitation in these patients. Local migration of neutrophils might be induced by the direct effect of tobacco contents,[18] and, however, additional findings suggested that cigarette smoke stimulated airway epithelial cells to release chemotactic factors for neutrophils such as IL-8.[19] In fact, the mRNA levels of IL-8 were increased in small airway epithelial cells from smokers as compared with nonsmokers.[20] Moreover, since IL-8 is also one of proangiogenic factors,[21] higher levels of IL-8 in small airways may be important in the development of small airways remodeling.

Based on the results of this study, we hypothesized that neutrophilic inflammation in the peripheral airways appears to play an important role in the pathogenesis of smoking-induced airway dysfunction. Future studies of the pathogenesis of smoking-induced airway dysfunction would focus on the accumulation and activation of neutrophils stimulated by IL-8 in the peripheral airways. However, there are several limitations in this study to reinforce our results. First, this preliminary study had a limited sample size. Second, a group of healthy nonsmoking subjects to clarify the direct effects of smoking were not included for an ethical reason. However, our new method in ELF sampling technique seems to avoid the difficulties in ELF measurement inherent in estimation of ELF from BAL fluid sampling or induced sputum. An accurate ELF measurement in the lungs will be potentially important area for a number of reasons including pathophysiological studies of various pulmonary diseases and assessment of pharmacological treatments. In summary, a less invasive and quantitative bronchoscopic microsampling technique was newly explored to measure biochemical constituents in ELF separately from central or peripheral airways. Using this new method, we for the first time showed increased levels of IL-8 in ELF from peripheral airways of smokers with airflow limitation compared with those without airflow limitation. These findings may shed new light on the pathogenesis of smoking-induced airway dysfunction. It is also clear that IL-8 in peripheral airways could be an interesting target for new pharmacological treatments in smokers with airflow limitation.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgments

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